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HIV-1 genetic diversity and drug resistance among treatment naïve patients from Southern Brazil: An association of HIV-1 subtypes with exposure categories

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ABSTRACT

Background: The AIDS epidemic in Southern Brazil has unique features, showing co-circulation of HIV-1 subtypes C, B and recombinant forms. Florianópolis has the second highest AIDS incidence among Brazilian capitals, but limited information is available about HIV molecular epidemiology and prevalence of primary drug resistance.
Objectives: To investigate the molecular epidemiology of HIV-1 in Florianópolis and to describe the prevalence of primary HIV-1 drug resistance mutations (DMRs).
Study design: Epidemiological and clinical data from 82 untreated patients from Florianópolis (2008–2009) were analyzed. The HIV-1 subtype at envelope, protease, reverse transcriptase and integrase regions were determined by phylogenetic and bootscaning analyses and the drug resistance profile were analyzed at the Stanford HIV Drug Resistance Database.
Results: The most frequent HIV-1 genetic form was subtype C (65.8%) followed by mosaics BC (18.3%), subtype B (13.4%), subtype F1 (1.2%) and BCF1 recombinant (1.2%). HIV-1 subtype C and BC recombinants were much more frequent in the heterosexual exposure category, whereas subtype B was more common in the MSM exposure category. DRMs were seen in 11% of the sequences, 2.4% of them were related to PI, 5% to NRTI, 3.6% to NNRTI and 1.2% was related to INTI.
Conclusions: The present study confirms the high prevalence of subtype C and BC recombinants in Santa Catarina State and revealed a significant difference in the subtype distribution among distinct virus exposure categories. This study also shows a relative high prevalence of protease/reverse transcriptase primary drug resistance mutations and corroborates the usefulness of the integrase inhibitors in southern Brazil.

1. Background

The HIV/AIDS pandemic has a major impact in Brazil, being the most affected Country in Latin America with over 600,000 people infected.[1] The Brazilian HIV-1 epidemic can be divided in two distinct scenarios. While subtype B prevails in most of the Country, with a secondary occurrence of subtype F1 and BF1 recombinants,[2–9] Southern Brazil presents a distinct pattern with high frequency of subtypes C, B and BC recombinants, following a lower proportion of subtype F1 and BF1 recombinants,[10–17] comprising 19% of the infected individuals in the Country.

The universal access of Brazilian HIV-positive patients to free-of-cost licensed antiretroviral drugs has substantially reduced the AIDS-related mortality.[1] The effectiveness of antiretroviral therapy, however, may be limited by the increasing of resistance to antiretroviral drugs and by transmission of such drug-resistant viruses to drug-naïve patients. National surveys suggest that primary HIV resistance in Brazil is generally low, ranging from 6% to 8%.[2,18,19] However, very high prevalence of HIV primary drug resistance (18–37%) were observed in some localities, such as the City of Santos (Southeastern region)[20,21] and the State of Bahia (Northeastern region),[22] indicating important differences across country regions.

Most of the molecular epidemiology studies on HIV in the Southern region were carried out in the State of Rio Grande do Sul, but

Abbreviations: AIDS, acquired immune deficiency syndrome; ARV, antiretroviral; CPR, calibrated population resistance tool; CRF, circulating recombinant form; DRMs, drug resistance mutations; INT, integrase; INTI, integrase inhibitors; HIV, human immunodeficiency virus; MSM, men who have sex with men; NJ, neighbor-joining; NNRTI, non-nucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; PCR, polymerase chain reaction; PI, protease inhibitors; PR, protease; RT, reverse transcriptase; URF, unique recombinant form.

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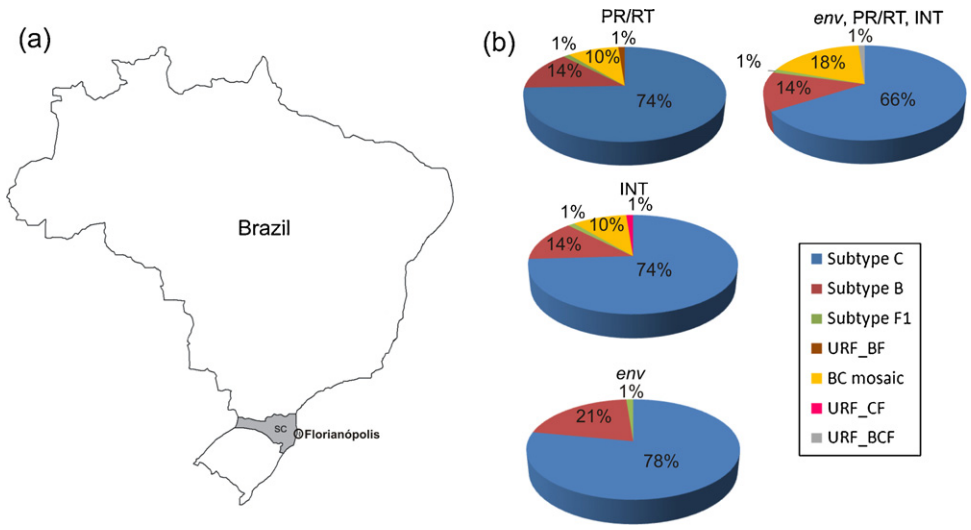

Fig. 1. Geographical location of Florianópolis and frequency of HIV-1 subtypes at the different genomic regions analyzed. (a) Map of Brazil highlighting the state of Santa Catarina (SC) and the metropolitan region of Florianópolis. (b) HIV-1 subtypes were determined by phylogenetic and bootscaning analyses, as described in Section 3.

limited data concerning the prevalence of HIV-1 subtypes and HIV primary drug resistance in the other southern states (Santa Catarina and Paraná) is available. Noteworthy, Santa Catarina State ranks third on AIDS incidence rate in Brazil (29.6 new cases per 100,000 inhabitants in 2008), while its capital, Florianópolis city, occupies the second position in the ranking of capitals (53.7 new cases per 100,000 inhabitants in 2008).[1] Moreover, Florianópolis city is a very important tourist pole in Southern Brazil, receiving people from other South American countries, mainly Argentina and Uruguay, which could have implications on the distribution of HIV subtypes and recombinant forms.

2. Objectives

This study describes the HIV-1 genetic diversity among different exposure categories and the prevalence of HIV primary drug resistance mutations based on sequence analyses of protease, reverse transcriptase, integrase and envelope genes among naïve patients from Florianópolis metropolitan area, Santa Catarina, Brazil.

3. Study design

3.1. Patients

Blood samples were collected from 82 HIV-infected patients followed up at Hospital Homero de Miranda Gomes located in metropolitan region of Florianópolis, Santa Catarina, Brazil. The individuals were selected from May-2008 to February-2009 and inclusion criteria included age over 18-year old, no sign or symptom of AIDS and no previous antiretroviral therapy. The Universidade Federal de Santa Catarina Ethics Committee approved the study and informed consent was obtained from all volunteers.

3.2. Extraction, amplification and sequencing of HIV-1 DNA

PBMC was isolated from 4 ml of blood by the Histopaque (Sigma–Aldrich, St. Louis, MO, USA) density gradient method and genomic DNA was extracted using a QIAmpl Blood Kit (Qiagen Inc., Chatsworth, CA, USA). HIV-1 protease (PR), reverse transcriptase (RT), integrase (INT) and envelope (*env*) genes were

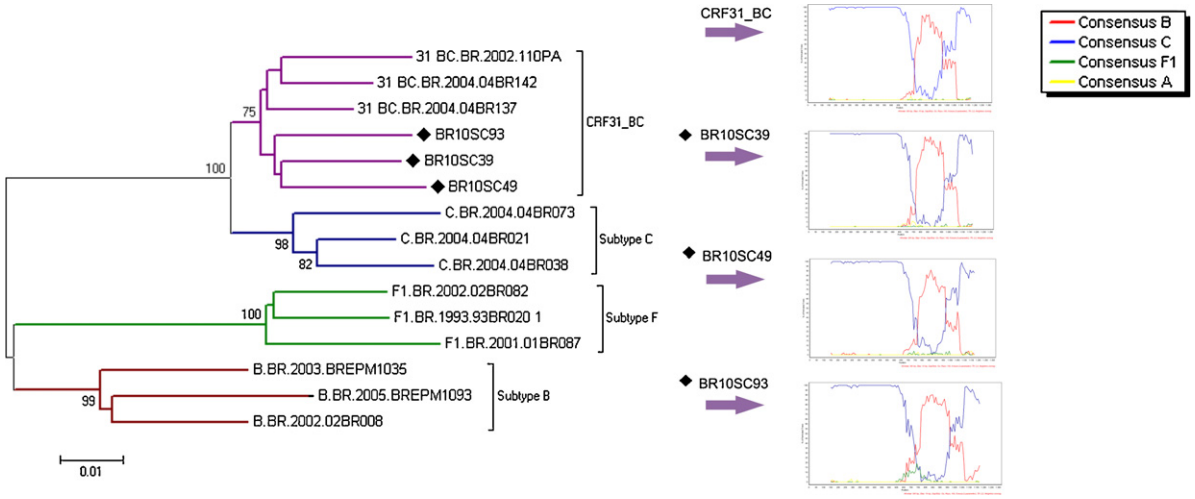

Fig. 2. Phylogenetic analysis of CRF31_BC-like sequences. (a) Neighbor-joining phylogenetic tree of PR/RT region (2316–3316 nt relative to HXB2), including only the CRF31_BC-like sequences from Santa Catarina (black diamonds) and subtype reference sequences. Reliability was tested with 1000 replicates bootstrapped, values considering significant (above 70%) are shown. (b) Bootscaning patterns corresponding to a CRF31_BC reference sequence and the three sequences from Santa Catarina classified as CRF31_BC like. Reference sequences were extracted from HIV Los Alamos Database.

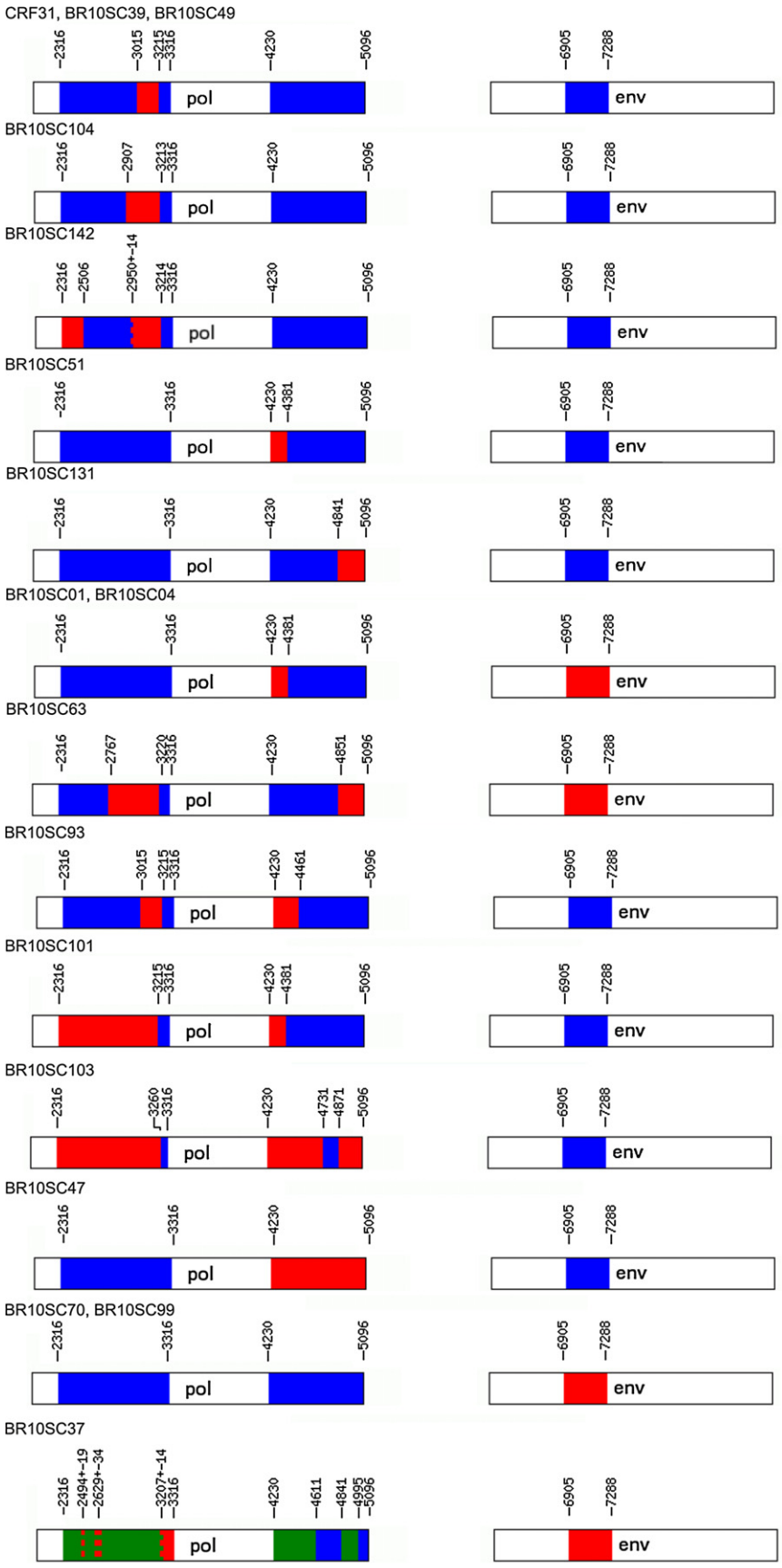

Fig. 3. Schematic drawing showing breakpoint pattern of the mosaics viruses found in the study. Breakpoint positions were obtained using Simplot 3.5.1 and numbered according to HXB2 reference. The *pol* (PR/RT, INT) and *env* (*gp120-C2V3*) genes are colored according to the subtype: subtype B is red, subtype C is blue and subtype F1 is represented in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
General characteristics of HIV-1 infected individuals according to the HIV-1 subtypes.

	Total (n = 82)	Subtype C (n = 54)	Subtype B (n = 11)	BC mosaic (n = 15)
Mean age (years)	37.8 ± 10	39 ± 9.2	31.3 ± 9.3	37 ± 11
Sex (%)				
Male	40 (48.8%)	23 (43%)	9 (82%)	7 (46.7%)
Female	42 (51.2%)	31 (57%)	2 (18%)	8 (53.3%)
Mean diagnostic year	2004 ± 3.8	2004 ± 4.3	2005 ± 2.1	2005 ± 2.7
Exposure categories				
Heterosexual	58 (70.6%)	41 (76%)	3 (27.3%)	12 (80%)
MSM	18 (22%)	9 (16.5%)	8 (72.7%)	1 (6.6%)
Injection drug user	4 (5%)	3 (5.5%)	–	1 (6.6%)
Unknown	2 (2.4%)	1 (2%)	–	1 (6.6%)
Mean CD4 T-cell count	513 ± 312	492 ± 269	563 ± 429	522 ± 366
Mean viral load (log_{10} copies/ml)	4.1 ± 0.8	4.1 ± 0.8	4.4 ± 0.9	3.9 ± 0.7

Values are expressed ± standard deviation of the mean.

amplified by a nested-PCR as described elsewhere.[23–25] Purified products were sequenced using MegaBace® 1000 DNA Analysis System (GE Healthcare). Sequences were visually inspected and assembled by using SeqMan software, LaserGene package (DNASTAR, Madison, WI, USA). Sequences were submitted to GeneBank under accession numbers JF773818-JF774062.

3.3. HIV-1 subtype classification

Nucleotide sequences were aligned by using Clustal X program.[26] The two *pol* gene alignments cover almost whole PR and part of RT (nucleotides 2316–3316 relative to HXB2) and whole INT (nucleotides 4230–5096 relative to HXB2), while the *env* gene alignment covers *gp120-C2V3* region (nucleotides 6905–7288 relative to HXB2). Phylogenetic analyses were performed by the Neighbor-Joining (NJ) algorithm using the Tamura-Nei nucleotide substitution model,[27] in 1000 bootstrap replicates, as implemented in MEGA v4 program.[28] Analysis of recombination was performed by bootscaning analysis (sliding window of 250 bp, incremental steps of 10 bases, and the Kimura two-parameter model[29]) using Simplot 3.5.1 software.[30] Bootstrap support was calculated based on 100 re-samplings.

3.4. Resistance analysis

Drug-resistance mutations (DRMs) profile and antiretroviral (ARV) susceptibility were inferred by submitting the PR/RT sequences to the Calibrated Population Resistance Tool (CPR) (version 5.0 beta; available at http://cpr.stanford.edu/cpr/servlet/CPR) and the INT sequences to the Stanford HIV Resistance Database (http://hivdb.stanford.edu/). The CPR program provides a standard approach to estimating the prevalence of transmitted HIV drug resistance using population-sampled sequence data and disregard natural occurring polymorphisms and mutations occurring in a frequency higher than 0.5% in untreated subjects.[31,32]

3.5. Statistical analysis

The Chi-squared test was employed to evaluate possible association of HIV-1 subtype with demographic, exposure category and clinical variables. A p value <0.05 was considered significant.

4. Results

Out of 82 HIV-1 isolates analyzed at the PR/RT, INT and *env* regions, 54 (65.8%) were classified as subtype C, 15 (18.3%) as B/C inter-subtype recombinants, 11 (13.5%) as subtype B, one (1.2%) as subtype F1 and one (1.2%) as B/C/F mosaic form (Fig. 1). Three (3.6%) BC recombinant sequences displayed a CRF31_BC-like recombination profile at PR/RT and clustered among CRF31_BC reference sequences in the *pol* (PR/RT) NJ tree (Fig. 2). One of these sequences (BR10SC93) displayed one additional B/C recombination point at INT and was classified as a unique recombinant form (URF), as well as the remaining inter-subtype recombinant sequences (Fig. 3).

Table 1 summarizes the demographic characteristics, exposure category, clinical and genetic HIV subtype data of the 82 patients analyzed. Forty (48.8%) patients were males and 42 (51.2%) were females, with a mean age of 37.8 years. The major exposure category was heterosexual (70.6%), followed by men who have sex with men (MSM) (22%) and injection drug users (5%). The herein presented data shows a highly significant association (p < 0.001) between exposure category and HIV-1 subtype. Eighty percent of

Table 2
Primary drug resistance mutations associated to protease, reverse transcriptase and integrase genes among treatment naïve patients.

Sample	*Pol* subtype	PI resistance mutations	NRTI resistance mutations	NNRTI resistance mutations	INTI resistance mutations	Resistance profile		
						Low	Intermediate	High
BR10SC06	C				S147R + E138A	RAL, ELV		
BR10SC07	C	N88D					NFV	
BR10SC29	B		K219Q			AZT		
BR10SC42	C	M46I					NFV	
BR10SC46	B		V75M			DDI	d4T	
BR10SC47	C			K101E, V106M, G190A, M230L			ETR	DLV, EFV, NVP
BR10SC50	C		M41L, T215C			ABC, DDI, TDF	AZT, d4T	
BR10SC107	C		M41L	G190A		AZT, d4T, ETR	EFV	NVP
BR10SC131	C			K103N				DLV, EFV, NVP

PI, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; INTI, integrase inhibitors; ABC, abacavir; AZT, zidovudine; DDI, didanosine; DLV, delavirdine; d4T, stavudine; EFV, efavirenz; ETR, etravirine; NFV, nelfinavir; NVP, nevirapine, TDF, tenofovir; RAL, raltegravir; ELV, elvitegravir.

BC recombinants (URFs_BC + CRF31_BC-like) and 76% of subtype C sequences were amplified from individuals of the heterosexual exposure category, whereas 73% of subtype B sequences were in MSM exposure category (Table 1). Subtype B sequences represent 44% of viruses circulating in the MSM population, but only 5% of viruses circulating among heterosexual or female populations. No statistically significant differences at CD4 T cell count, RNA viral load or mean age were found among HIV-1 subtypes.

Among 82 HIV-1 *pol* sequences obtained from drug naïve patients, the presence of at least one DRM was observed in 9 (11.0%) patients (Table 2). DRMs to protease inhibitors (PI) were observed in two (2.4%) patients. Polymorphisms and mutations at protease secondary codons were seen in 17 (20.7%) sequences, being L10I/V and T74S the most frequently found (data not shown). Mutations related with resistance to nucleoside reverse transcriptase inhibitors (NRTI) were observed in four (5%) sequences, and M41L was the most common. DRMs to non-nucleoside reverse transcriptase inhibitors (NNRTI) were observed in three (3.6%) of the sequences analyzed, and G190A was the most common. Resistance to integrase inhibitors (INTI) was observed in only one (1.2%) patient who displayed the resistance mutations S147R and E138A, which confer a low-level of resistance to raltegravir and elvitegravir. Minor drug resistance mutations at INT were observed in 11 (13.5%) sequences, being V151I the most frequently found, especially among subtype B samples (data not shown).

One patient showed DRMs to more than one class of antiretroviral drugs. Among the 11 HIV-1 subtype B *pol* sequences, two (18%) displayed DRM, whereas among the 56 HIV-1 subtype C *pol* sequences, eight (14.3%) showed at least one DRM. Nevertheless, that difference was not significant ($p > 0.05$) and no correlation between resistance mutations and HIV-1 subtypes at *pol* was found.

5. Discussion

The present work describes a high prevalence (65.8%) of HIV-1 subtype C in Florianópolis metropolitan area, consistent with two recent studies which described a subtype C prevalence of 64% and 79% in other cities of Santa Catarina State.[13,16] This confirms the higher prevalence of subtype C in Santa Catarina when compared with other Southern Brazilian states: 20–30% in Paraná[15,17] and 27–42% in Rio Grande do Sul.[12,13] The molecular epidemiology scenario of Florianópolis also shows a high frequency (18.3%) of BC recombinants. Two out of 15 BC recombinant sequences identified showed a CRF31_BC-like recombinant pattern at *pol* (PR/RT/INT) and *env* genes, reinforcing that HIV-1 CRF31_BC circulates at low frequency in Santa Catarina, consistent with previous studies.[13,16] One URF_BC identified herein displayed a CRF31_BC-like pattern at PR/RT, but one additional B/C breakpoint at INT, while others URFs_BC displayed one coincident recombination breakpoint with CRF31_BC at PR/RT, but larger subtype B fragments. These variants may be second generation recombinants originated from recombination between CRF31_BC and subtype B strains, as was recently described in Rio Grande do Sul.[25] Although Florianópolis receives a very important number of tourists from Argentina and Uruguay, we found no evidence of circulation of BF recombinant forms commonly observed in those countries (such as CRF12_BF and CRF38_BF)[33], pointing to a minor impact of international tourism on the HIV molecular epidemiology scenario of Florianópolis.

A significant association was observed between HIV-1 subtype and exposure category, suggesting that two independent epidemics are occurring in Santa Catarina. It seems that HIV-1 subtype C broke up the barriers that separate the exposure categories and circulates at high prevalence within both heterosexual (70.7%) and MSM (50%) groups. By contrast, HIV-1 subtype B circulates at high prevalence within MSM group (44.4%), but not within heterosexual group (5.2%). On the other hand, the high prevalence of URFs_BC reflects frequent episodes of coinfections and/or superinfections in the studied population. The connection between subtypes B and C epidemics seems to be more frequent in heterosexual population, but further investigations with a higher number of MSM patients are necessary to confirm this observation.

Comparison of the HIV-1 *env* gene results from the current work with those obtained in a previous study performed in the same area[14] revealed a significant increase in the proportion of subtype C at heterosexual population from 56% in 2004 to 83% in 2008/2009; while the proportion of *env* subtype B decreases from 38% to only 15% in the same period. This result suggests a faster spread of subtype C than subtype B among heterosexual population over last years in Santa Catarina. These observations may reveal a different efficacy of homosexual/heterosexual transmission of subtypes B and C, or may reflect different entries and dissemination networks of these subtypes in Santa Catarina. The high proportion of subtype B among females in Rio Grande do Sul and Paraná (38–42%),[12,17] and the recent expansion of subtype C among MSM in São Paulo (Brazil),[34] strongly favor the second hypothesis.

Our findings indicate a prevalence of PR/RT primary DRMs (10%) higher than the national average (6–8%),[2,18,19] although lower than those described in other Brazilian regions (18–37%).[20–22] Notwithstanding, the prevalence of primary resistance presented herein may be underestimated due mostly selected patients were chronic cases (>12 months since HIV infection), and HIV tends to return to wild type over time without ARV selective pressure as DRMs reduce the viral fitness.[35] We also observed one patient with low-level resistance to INTI. Such resistance mutations, however, should not be considered a case of transmitted resistance since the diagnostic date of that patient was 2002, several years before the introduction of Raltegravir in Brazil (2009). Those mutations probably reflect rare substitutions in the integrase gene as described elsewhere.[36–38] These results encourage the use of this new drug in the treatment of AIDS patients in Southern Brazil.

In conclusion, our study indicates an increase of HIV-1 subtype C prevalence in the metropolitan area of Florianópolis with a significantly association between virus subtypes and exposure categories. The Southern region shows the highest incidence of HIV-1 infections in the country, which combined with the increasing contribution of HIV-1 infections among heterosexuals, might explain the expansion of subtype C in the Brazilian epidemic. This combination makes southern Brazil an interesting site for development of health innovations and vaccine trials.[39] The presented work also describes a prevalence of DRMs above the national average, pointing to the importance of sustained surveillance studies in this region.

Conflict of interest

Authors declare no conflict of interest.

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